Tips and Tricks for Multiplexing 5 or more probes.

In order to create a multiplex with 5 or more probes, it is imperative that the species of the antibodies, both primary and secondary, are selected carefully to prevent cross-reaction. Each primary antibody will have to be from a different species since they will be added on together at the same time. Fortunately, technology with secondary antibodies has improved drastically over the past few years and it is very reasonable to find a combination of secondary antibodies that will fit the primary antibody species, without cross-reacting with one another. The secondary antibodies should all be made in the same species. Most likely it will be optimal to choose donkey as your host since there are very few primary antibodies that are actually made up in donkey and most of the fluorophore options will come made in donkey. It is also critical that you purchase highly cross-adsorbed secondary antibodies to further reduce non-specific staining and cross reaction.

It is also important to consider the spectral overlap with the fluorophores. It is recommended that there is at a minimum, 20nm difference between the peaks of each fluorophore in order to be able to adequately spectrally unmix the sample. As far as where to purchase the best combinations of secondary antibodies, there are two companies that we primarily used: Invitrogen and Biotium. Both of these companies provide a multitude of secondary antibody options throughout the visible and IR spectra, with many species options. Biotium provides a cost-effective equal quality product for secondary fluorophores that tend to come in a wider array of species options. For our staining purposes, in order to get the best 7 colored images, we had to utilize secondary antibodies from both companies in conjunction with one another. From Biotium we used CF488, CF568, CF594, and CF633. From Invitrogen we used AF514 and AF660.

Alternatively to an indirect 2ndary antibody staining protocol, this same technique should be applicable using direct antibody conjugates if proper care is taken with the conjugations.

1. Slides baked at 65 degrees for 30-60 minutes.
2. The deparaffinization step was performed as described and demonstrated with the Leica Autostainer. Allow the slides to wash in the final wash step with running DI water for 5-10 minutes minimum.
	1. If there is a significant amount of autofluorescence it is advised to increase the times that the slides are in the ethanol washes, we have seen better results using this method.
3. Target retrieval was performed in the microwave. The buffer used was the Dako Target Retrieval Solution with a pH of 6. The slides were boiled in the buffer and maintained for 15 minutes.
	1. After allowing the slides to cool down in the buffer, for approximately 30-45 minutes, the buffer was gradually rinsed out using running DI water to slowly replace the buffer and wash off excess. Allow the slides to rinse in the running DI water for 5-10 minutes.
	2. The slides are then rinsed in a 1x TBS-T wash buffer 2 times for 5 minutes each on a rocker.
4. After rinsing, a PAP pen is used to draw hydrophobic circles around the area of interest. After the pen has been applied, the slides are put back into the wash buffer and allowed to sit for 5 minutes.
5. In a humidity chamber, the blocking buffer is added to each of the slides for 30-45 minutes gently on a rocker. The optimal blocking buffer we used was the Dako Serum-free protein block. Using a serum-free block allows easier and optimal subsequent staining steps when dealing with many species at a time.
	1. After blocking, the block buffer is removed using aspiration, be sure to not let the slides dry out at any point during these steps.
6. The primary antibodies are all going to be added at exactly the same time, all together. The antibodies were diluted according to the manufacturers recommendations, or to an optimal dilution if it needs to be adjusted. The antibodies are diluted in the Dako Antibody Diluent. The slides are incubated in a humidity chamber overnight at 4 degrees, gently on a rocker.
	1. Remember that a control slide with each combination of the primary and secondary antibodies needs to be performed alongside the slides receiving the full treatment, as well as a slide that has no antibodies.
7. After incubation, the antibodies are removed using aspiration. The slides are then washed in the wash buffer 3 times for 5 minutes each, on a rocker.
8. The secondary antibodies will also all be applied together at one time, all together. The antibodies are diluted in the Dako Antibody Diluent at 1:1000 dilutions. For the colors that are not as bright, such as the AF514 or AF660, a lower dilution, 1:500, may be required to get a brighter signal. The secondary antibodies are incubated in a humidity chamber, blocked from light, at room temperature for 45-60 minutes, gently on a rocker.
9. After the incubation, the antibodies are removed by aspiration. The sldies are then washed in the wash buffer 3 times for 5 minutes each, blocked from light, gently on a rocker.
10. DAPI, or an alternative nuclear stain, is then added to the slides receiving the full treatment and the control slides with only DAPI. Typically, a 1:10000 dilution for 60 seconds is sufficient; however, this may need to be altered depending on your DAPI concentration and type of DAPI.
	1. After incubation, the slides that received DAPI are washed in the wash buffer 3 times for 5 minutes each, blocked from light, gently on a rocker.
11. The slides are then coverslipped with a high quality glass coverslip. The thickness of the coverslip will depend on what is optimal for the objective that you will be using. Typically with high quality oil immersion objectives, the #1.5 coverslips are the optimal thickness. The mounting medium found to be the most useful and compatible with all of the used fluorophores was Prolong Gold. Be aware that some mounting mediums are not compatible with certain fluorophores.
	1. The slides are then sealed using a coverslip sealer. We use Biotium’s CoverGrip Coverslip Sealant. This is an organic, solvent free sealer that will not leach into your sample and interfere with fluorescence.

For the analysis side of this protocol there are only a few tips that will help produce better results.

1. Use the smallest step size possible for scanning and creating the cubes. We used 2nm step sizes to produce the best spectra.
2. Utilize the “narrow” feature for scanning the spectra if it is available with your system. This will provide tighter peaks for the spectra, which will allow better distinguishing between fluorophores close together in the spectra.
3. The best results will be obtained if you have the ability to use a multi filter system instead of longpass filters. A filter for each fluorophore will provide the best spectras of each fluorophore, allowing the software to determine the colors more accurately and unmix them. Since having a filter for each fluorophore may not be possible with your system, and can be rather expensive, a system optimally utilizing both narrow and longpass filters to properly excite and subsequently detect the emission of each fluorophore is a great alternative. We utilized a DAPI filter, a longpass GFP filter, a narrow GFP filter, a longpass mCherry filter, a narrow mCherry filter, and a TRITC filter to optimally cover the previously mentioned fluorophores. An Infrared filter can also be utilized to use fluorophores that are above 700nm.